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Phylogeography of a Rare Serpentine Plant, *Arenaria katoana* Makino (Caryophyllaceae)

DAIJU KAWASE[1*], TAKAKAZU YUMOTO[1] AND KEN SATO[2]

*[1]Research institute for Humanity and Nature, 457-7 Motoyama, Kamigamo, Kita-ku, Kyoto, 603-8047 Japan. *kawadai@chikyu.ac.jp (author for correspondence); [2]Faculty of Engineering, Hokkai-Gakuen University, Sapporo, Hokkaido 062-8605, Japan*

Arenaria katoana (Caryophyllaceae) is a rare endemic plant that occurs disjunctly on ultramafic rock sites in Japan. This study assessed the degree of genetic distinctness of small, disjunct, isolated populations of *A. katoana* in Japan. We sequenced several non-coding regions of the chloroplast DNA (*trnL* intron, *trnL–trnF* intergenic spacer, *psbA–trnH* intergenic spacer, and *petG–trnP* intergenic spacer) and nuclear ribosomal DNA (ITS1, 5.8S nrDNA, and ITS2) to determine the relationships between populations and to evaluate the level of intraspecific genetic variation. Eight different chloroplast DNA haplotypes were detected, and each population exhibited a unique cpDNA haplotype. Four different ITS types, which were shared between populations, were detected. The phylogeny and distribution patterns of the cpDNA haplotypes and the ITS types indicated that *A. katoana* can be divided into three groups; a central Hokkaido group (Mt. Yubari, Mt. Bozu, and Mt. Furano-nishi-dake), a Mt. Apoi and Mt. Shibutsu group, and a Mt. Hayachine group. The populations of *A. katoana* have been subjected to the effects of genetic drift and restricted gene flow, both of which promote population differentiation.

Keywords: *Arenaria katoana*, cpDNA, ITS, rare plant, serpentine

Ultramafic areas may have adverse edaphic effects on vegetation due to the presence of large amounts of Nickel (Ni) and Magnesium (Mg), the xeric nature of the environment, and stochastic disturbances caused by landslides (Proctor & Woodell 1975, Chiarucci *et al.* 2001, Mengoni *et al.* 2003b, Berglund *et al.* 2004). At the same time, the large number of endemic species that occur on such sites suggests ultramafic soils are hotspots of biological diversity (i.e., centers of floristic differentiation and speciation; Stevanović *et al.* 2003).

Arenaria katoana Makino (Caryophyllaceae) is a remarkable serpentine alpine plant that is distributed disjunctly on ultramafic (serpentine and peridotite) sites in Japan. It is considered to be a serpentine relict species (ultrabasicosaxicolous relic; Makino 1908, Kitamura 1950, Toyokuni 1955). *Arenaria katoana* var. *lanceolata* on Mt. Apoi, which is known for its large number of endemic species, is recognized as distinct from typical *A. katoana* on the basis of its narrow leaves (Tatewaki 1928).

Populations of *Arenaria katoana*, one of the rarest species in Japan, have decreased by about 30% over the last 10 years (Environmental Agency of Japan 2000) and is thus officially recognized as a vulnerable species (VU rank). *Arenaria. katoana* is currently limited to open, ultramafic rock sites located around Mt. Yubari, Mt. Tottabetsu, Mt. Apoi, Mt. Furano-nishi-dake, and Mt. Bozu in Hokkaido, Mt. Hayachine in Iwate Prefecture, and Mt. Shibutsu and Mt. Tanigawa in Gunma Prefecture. The number of individuals in each population is very small.

It is necessary to evaluate the genetic diversity of endemic serpentine plants with narrow distributions, as small isolated populations are highly susceptible to loss of genetic variation due to genetic drift. Information on the level of intraspecific genetic variation in rare species is vital to the identification of appropriate units for conservation (Petit *et al.* 1998, Newton *et al.* 1999). Moreover, ecological and genetic information on serpentine plants can be used to assess how the plants have adapted to, and evolved in, such a stressful environment.

In this study, we investigated the genetic variation and phylogeography of *A. katoana* using sequence information collected from chloroplast (cp) DNA and internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA). Four non-coding regions (*trnL* intron, *trnL–trnF* intergenic spacer [IGS], *psbA–trnH* IGS, and *petG–trnP* IGS) in cpDNA and the internal transcribed spacer (ITS) regions ITS1, 5.8S, and ITS2 in the nrDNA were used to determine the extent of contemporary genetic variation.

Materials and Methods

Plant materials

Forty one leaf samples were collected from the six the major remaining localities of *Arenaria katoana* var. *katoana* on Honshu and Hokkaido and *A. katoana* var. *lanceolata* on Mt. Apoi, Japan, in 2004 and 2005. The location of the sites is indicated in Table 1 and Fig. 1.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from silica-gel-dried leaves (20–50 mg) using a DNA extraction kit (Plant Genomic DNA Extraction Miniprep System; Viogene, Sunnyvale, CA) according to the manufacturer's protocol.

PCR was performed in 20 μL volumes containing 50–100 ng template DNA, 2 μL of 10X PCR buffer (TOYOBO, Japan), 0.2 mM of each deoxyribonucleotide, 0.2 μM of each of the primer pairs, and 0.5 U of BlendTaq DNA polymerase (TOYOBO, Japan). Amplification was performed using the GeneAmp PCR System 9700 (Applied Biosystems, USA).

The four non-coding regions the cpDNA, were amplified by using the following universal primers: (1) "c" and "d" in *trnL* intron and (2) "e" and "f" in *trnL–trnF* (Taberlet *et al.* 1991), (3) "psbA" and "trnH (GUG)" in *psbA–trnH* (Hamilton 1999), and (4) "petG" and "trnP" in *petG–trnP* (Hwang *et al.* 2000). Each PCR cycle followed the same program: 30 cycle reaction with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, in addition to initial denaturation at 95°C for 3 min and a final extension at 72°C for 2 min.

For amplification of the ITS regions, the PCR cycle proceeded according to the following program: 30 cycle reaction with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, in addition to initial denaturation at 94°C for 3 min and final extension at 72°C for 2 min. The ITS region was amplified using universal primers combination (ITS5 and

TABLE 1. Location of study sites including cpDNA haplotypes and ITS types found at each site. Numbers in parentheses indicate number of cpDNA haplotypes in each population (refer to Fig. 1).

Locality	Latitude (E)	Longitude (N)	Altitude (m)	Number of individuals analyzed	cp DNA Haplotype	ITS type
Mt. Shibutsu, Gunma Pref.	36.54	139.10	2228	8	A	a
Mt. Hayachine, Iwate Pref.	39.33	141.29	1344	12	B(8), C(4)	b
Mt. Apoi, Hokkaido (*A. katoana* var. *lanceolata*)	42.06	143.01	810	10	D(2), E(8)	a
Mt. Bozu, Hokkaido	42.50	142.12	790	3	G	d
Mt. Yubari, Hokkaido	43.06	142.14	1667	6	F	c
Mt. Furano-nishi-dake, Hokkaido	43.18	142.18	1331	2	H	c

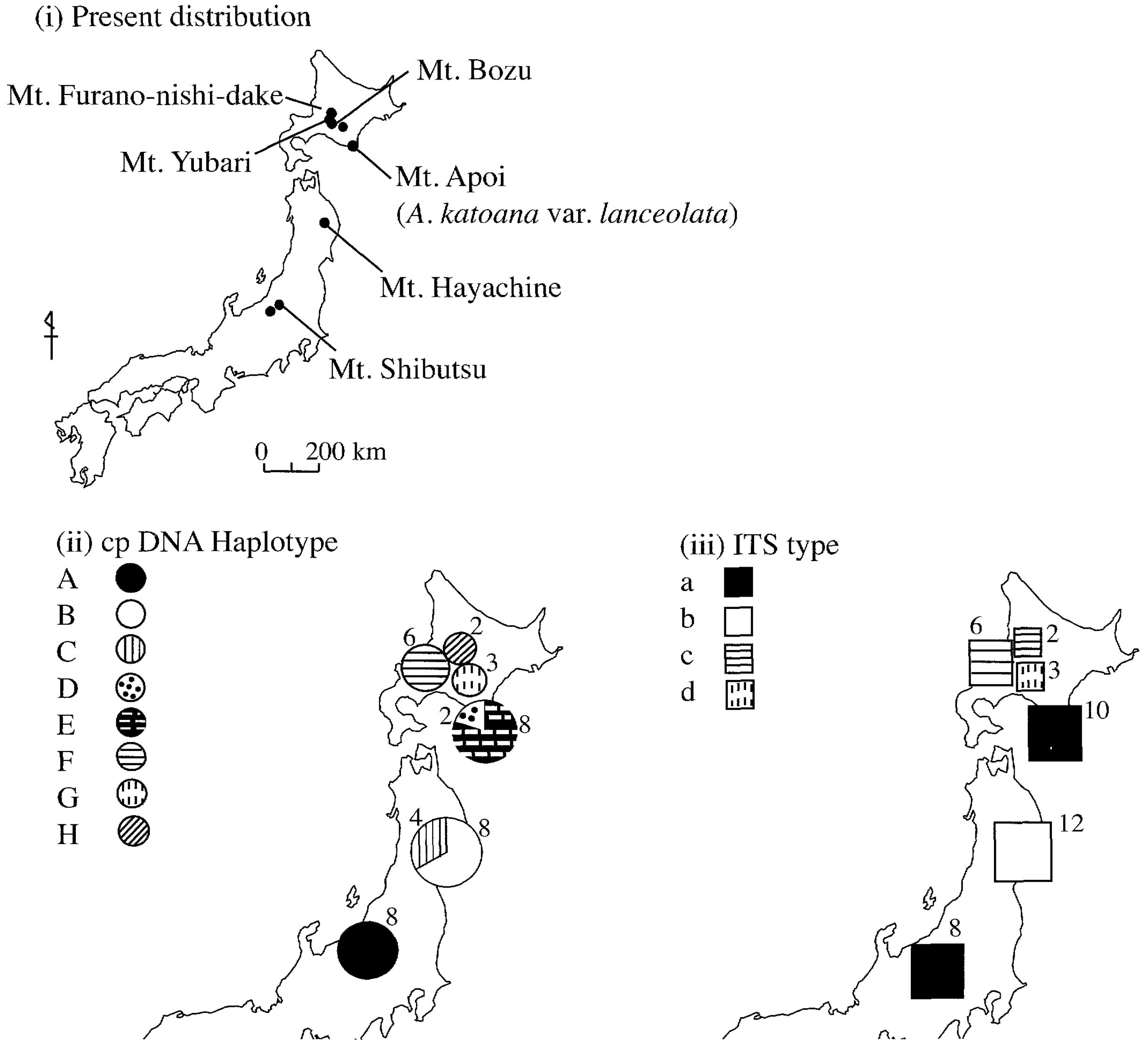

FIG. 1. (i) Distribution of *A. katoana*. Location names indicate populations analyzed and listed in Table 1. (ii) Distribution of cpDNA haplotypes. (iii) Distribution of ITS types. Letters correspond to cpDNA haplotypes and ITS types in Tables 2 and 3. Numbers above circles and squares correspond to number of individuals analyzed.

TABLE 2. Variable sites of aligned sequences of cpDNA regions of *trnL intron*, *trnL–trnF*, *psbA–trnH*, and *petG–trnP*. Numbers in parentheses indicate number of repeated mononucleotides.

Region (length)	*trnL* intron (583–585 bp)					*trnL–trnF* (323–324 bp)		*psbA–trnH* (313 bp)	*petG–trnP* (375 bp)
Variable sites	2	2	3	4	4		1	1	
	3	8	2	2	2	6	6	0	5
cp DNA Haplotype	4	8	0	2	4	9	4	1	0
A	A	T(10)	A	A	C	C(8)	T	G	A
B	A	T(8)	A	A	A	C(7)	T	T	C
C	A	T(8)	A	A	A	C(7)	G	T	C
D	A	T(10)	C	A	A	C(8)	T	G	A
E	A	T(10)	A	A	A	C(8)	T	G	A
F	T	T(9)	A	A	A	C(8)	T	T	A
G	A	T(9)	A	A	A	C(8)	T	T	A
H	T	T(9)	A	C	A	C(8)	T	T	A

ITS4), and one of the internal sequencing primers combinations (ITS5 and ITS2, ITS3 and ITS4) for degraded DNA (White *et al.* 1990).

The amplified fragments (20 μL) were treated with 4 μL of 1/20 diluted ExoSAP (GE Healthcare Bio-Sciences KK) solution to remove excess primers and dNTPs. Fragments were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with the primers described above. We analyzed the sequences using an ABI3100 sequencer with the Sequencing Analysis 3.7 program (Applied Biosystems, USA).

Statistical analysis

Multiple alignments of the sequences were obtained using clustal W software (Thompson *et al.* 1994). Each gap of the sequenced regions was treated as a fifth character state. A haplotype network was built by using the computer program TCS 1.21 and estimation of the 0.95 probability limit for parsimony and the network connections was obtained (Clement *et al.* 2000). As the parameters of genetic diversity and populations differentiation, total (*hT*) and within-population diversity (*hS*), and *Gst* and *Nst*, were estimated following the methods described by Pons & Petit (1995, 1996) using the programs HaploNst (http://www.pierroton.inra.fr/genetics/labo/Software/). In the estimated two parameter, G_{ST} is based only on haplotype frequencies, while N_{ST} takes into consideration genetic similarities between haplotypes and establishes the extent to which mutational differences between haplotypes contributes towards population differentiation. The differences in these two parameters were compared by a permutation test using 1000 permutations (Burban *et al.* 1999).

Results

Sequence variations

Eight haplotypes (A–H) were detected in the cpDNA sequences. The following variations were identified: the *trnL* intron (583–585 bp) contained four substitutions and a length polymorphism of mononucleotide repeats (poly-T), *trn L–trnF* IGS (323–324 bp) contained one substitution and a length polymorphism of mononucleotide repeats (poly-C), and *psbA–trnH* IGS (313 bp) and *petG–trnP* IGS (375 bp) each contained one substitution (Table 2). In multiple alignments of the *trnL* intron, *trnL–trnF* IGS, *psbA–trnH* IGS, and *petG–trnP* IGS, proportions of polymorphic sites including gaps were 1.03%, 0.62%, 0.32%, and 0.27%, respectively. The *trnL* intron had the highest level of genetic variation.

Four ITS types (a–d) were detected in the ITS sequences. The ITS region, which was 623 bp long and contained no indels, included ITS1 (247 bp), 5.8S nrDNA (145 bp), and ITS2 (231 bp). ITS1 contained four substitutions, while ITS2 had two substitutions (Table 3). In multiple alignments of ITS1, 5.8S nrDNA, and ITS2, proportions of polymorphic sites were 1.62%, 0.00%, and 0.87%, respectively. The ITS region sequenced directly from PCR products did not show nucleotide polymorphisms within individual samples. The 18 sequences of the four cpDNA regions and the ITS were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB433180–AB433196 and AB433631.

TABLE 3. Variable sites of aligned ITS sequences.

Region (length)	ITS1 (247 bp)				ITS2 (231 bp)	
Variable sites	 8 8	1 0 2	1 6 3	2 0 4	1 7 9	2 0 7
ITS type						
a	C	G	G	C	C	C
b	C	G	A	C	T	A
c	T	G	A	T	C	C
d	T	A	A	T	C	C

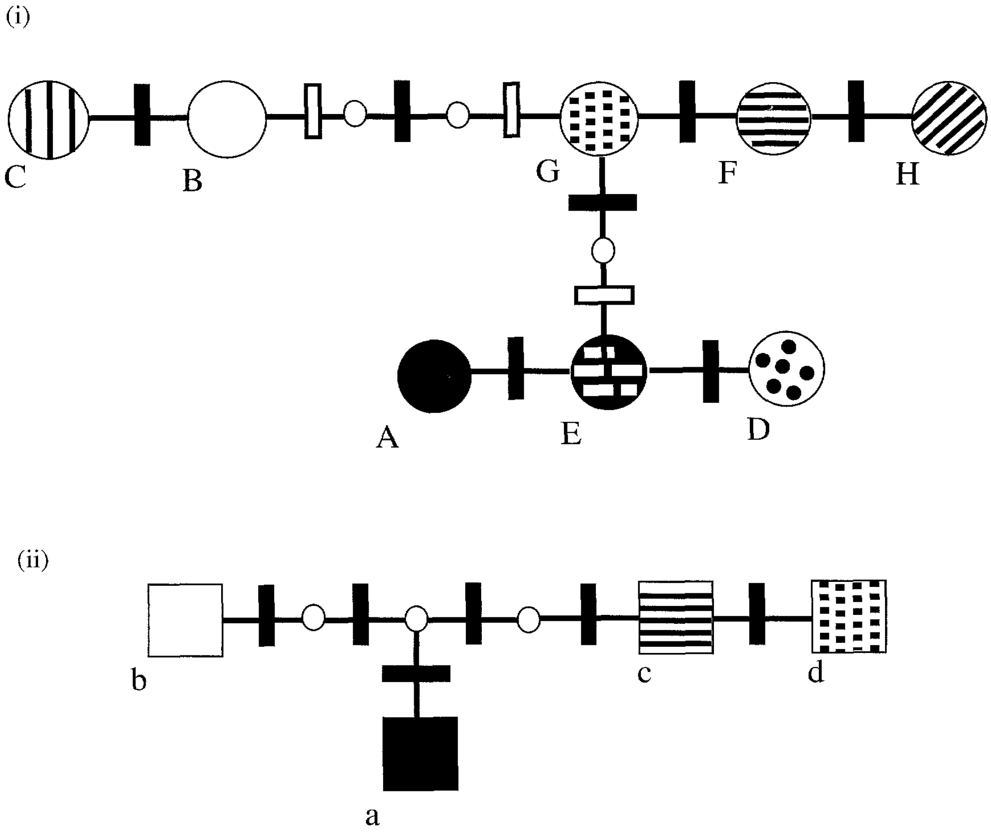

FIG. 2. Parsimonious networks of cpDNA haplotypes (i) and ITS types (ii) constructed using TCS program. Small circles indicate missing haplotypes. Solid and open bars represent nucleotide substitutions and length polymorphisms, respectively.

Geographical distribution and phylogenetic relationships of the cpDNA haplotypes and the ITS types

The geographical distribution of the cpDNA haplotypes and their ITS types are shown in Fig. 1. The phylogenetic relationships of the cpDNA haplotypes and ITS types are shown in Fig. 2. Our results revealed that the eight cpDNA haplotypes (type A–H) were restricted to specific populations. Mt. Apoi had two cpDNA haplotypes (D, E), which were phylogenetically close to those found on Mt. Shibutsu (A). Mt. Hayachine also had two haplotypes (B, C) that were phylogenetically close to each other. The other populations had only one cpDNA haplotype each. The populations in central Hokkaido (Mt. Yubari, Mt. Furano-nishi-dake, and Mt. Bozu) had phylogenetically close haplotypes (F, G, and H). The total diversity (*hT*) and the within-population diversity (*hS*) of cpDNA was 1.000 and 0.140, respectively. *Gst* and *Nst* of cpDNA were 0.860 and 0.958, respectively. The difference between the values of *Gst* and *Nst* was significant at the 5% level from a permutation test.

Mt. Apoi had the same ITS types (a) as Mt. Shibutsu, and Mt. Hayachine had one ITS type (b). The populations in central Hokkaido (Mt. Yubari, Mt. Furano-nishi-dake, and Mt. Bozu) had phylogenetically close ITS types (c and d). The total diversity (*hT*) and within-population diversity (*hS*) of the ITS was 0.897 and 0.089, respectively. *Gst* and *Nst* of the ITS were 0.897 and 0.906, respectively. The values of the two parameters were not significantly different ($P = 0.458$).

Discussion

Phylogeographical patterns of cpDNA and ITS variations

Mt. Yubari, Mt. Furano-nishi-dake, and Mt. Bozu, which are all located close to the center of Hokkaido, had phylogenetically close cpDNA haplotypes (F, H, and G) and ITS types (c and d). Mt. Shibutsu and Mt. Apoi had phylogenetically close cpDNA haplotypes (A, D, and E), and the same ITS type (a). Mt. Hayachine had phylogenetically different cpDNA haplotypes (B and C) and ITS type (b) compared to other regions. Thus, *A. katoana* can be phylogenetically divided into three groups: the central Hokkaido group (Mt. Yubari, Mt. Bozu, and Mt. Furano-nishi-dake), the Mt. Apoi and Mt. Shibutsu group, and the Mt. Hayachine group.

Genetic variation of alpine plants in Japan has recently been studied with respect to their individual habitats, and the effects of climate change on plant distribution and colonization routes have been revealed (Fujii *et al.* 1995, 1996, 1997, Ikeda *et al.* 2006, Ikeda & Setoguchi 2006). These studies have suggested the importance of the mountains in central Honshu as refugia during the Quaternary climatic oscillation, and that alpine plants have had their own histories and genetic structure. In the case of *A. katoana*, the geographical distribution and phylogenetic relationships of the cpDNA haplotypes suggest significant phylogeographic structure within the entire distribution range ($Nst > Gst,\ P < 0.05$). In the ITS types, we observed no significant phylogeographic structure. The populations on Mt. Shibutsu, which is in central Honshu, had low genetic diversity, with only a single cpDNA haplotype. The northern populations on Mt. Apoi and Mt. Hayachine had two cpDNA haplotypes. The low haplotype diversity and the unique cpDNA haplotypes in each population may reflect genetic drift due to the effect of the small population sizes. The amount of genetic diversity within each population is low, and most of the existing diversity can be explained by a between-population diversity component. In a previous population genetic and phylogenetic study of the endemic serpentine plant *Calystegia collina* (Convolvulaceae) high genetic differentiation and low gene flow between populations was found, not just between small outcrops but also between populations occupying the same large outcrop (Wolf *et al.* 2000). Furthermore, populations of another endemic serpentine plant, *Alyssum bertolonii* (Brassicaceae), were reported to be established by a small number of founders, with each population determined to be a distinct genetic entity, reflecting a long history of spatial isolation between individual populations and serpentine sites (the “ecological island model”; Lefèbvre & Vernet 1990, Mengoni *et al.* 2003a). The current geographical distribution of *A. katoana* is extremely narrow and restricted to serpentine sites; moreover, the populations are small in size. Thus, the populations of *A. katoana* have been subjected to the effects of genetic drift and restricted gene flow, both of which promote population differentiation.

In terms of conservation genetics, the three phylogenetic groups, Central Hokkaido, Mt. Apoi and Mt. Shibetsu, and Mt. Hayachine, can be recognized as independent units for protection. Additionally, different populations within each phylogenetic group also contain unique cpDNA haplotypes. A conservation program for *A. katoana* should therefore be planned based on this phylogeographic information.

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